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# PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF SOYBEAN (SOJA MAX PIPER) AND OF FACTORS INFLUENCING NODULE PRODUCTION

## A THESIS

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF CORNELL UNIVERSITY FOR THE DEGREE OF

## DOCTOR OF PHILOSOPHY

BY

JAMES KENNETH WILSON

JANUARY, 1917

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# PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF SOYBEAN (SOJA MAX PIPER) AND OF FACTORS INFLUENCING NODULE PRODUCTION

J. K. Wilson

From a consideration of the literature of the subject it becomes apparent that there is a lack of fundamental information with respect to the morphological and physiological characteristics of the bacteria causing nodule production in leguminous plants, and that no thoro investigation has been made of the factors controlling nodule production. Moore (1905)[1] has stated that these factors vary with each member of the legume family and depend on the requirements of the host plant. In order to increase the knowledge of this subject, the writer has concluded investigations on the organism causing nodule production on soybeans and the factors influencing nodule production on this host. The soybean organism was selected for investigation because of its distinctive cultural characteristics and because of the increasing importance in New York State of the soybean as a crop.

The investigation includes study of the following points:

1. Morphological and physiological characters of the organism causing nodule production on soybeans.

2. Factors that may influence the formation of nodules on soybeans grown in soil or water cultures, using not only the salts that are essential for the development of the plants but also non-nutrient salts and various other substances. An effort was made to determine also the relation of the soil moisture content to the formation of nodules.

3. Vitality of *Bacillus radicicola* of soybean under different cultural conditions. An attempt was made here to determine whether or not inhibition of nodule production is due to death of the organism or to other causes.

4. Time required for infection. This includes, by indirect evidence, the time required for the organism to gain entrance into the root.

5. Is nodule production associated with the nitrogen requirement of the plant?

## REVIEW OF LITERATURE

Relatively little work has been done to determine the influence of various substances on nodule formation, despite the fact that over

[1] Dates in parenthesis refer to bibliography, page 412.

sixty years ago Rautenberg and Kühn (1864), in investigations with *Vicia Faba*, incidentally observed that no nodules were formed on the plant roots when they were grown in various solutions containing nitrates.

No more observations are noted until De Vries (1877) presented the results of his work with red clover. He found also that when plants were grown in full nutrient solutions containing nitrates at certain concentrations, the formation of nodules was inhibited.

Vines (1888–89), experimenting with *Vicia Faba*, employed soil cultures with boxes as the containers, and grew the plants both in the greenhouse and in the open. The beans were soaked in a nitrate solution before planting, and the solution was added to the soil after the beans were planted. Vines examined the roots after pulling up the plants. He concluded that the production of nodules was much less when a nitrate was present in the soil than when it was absent. His work indicates also that as the supply of nitrates decreases the number of nodules increases, and he advanced the idea that nodule production is associated with certain conditions of nutrition.

The same year Frank (1889) grew lupines and peas, on the one hand in a humous soil and on the other hand in a humus-free soil, and observed that nodules were freely formed in the latter case but none were produced in the former.

The effect of moisture on nodule production was noted by Gain (1893). He grew *Pisum sativum*, *Lupinus albus*, and *Faba vulgaris* in an arid section, watering some of the plants and leaving the remainder unwatered. Not only did the watered plants have a larger number of nodules, but the nodules were differently located. The beans that were watered produced several times as many nodules as did those that were left unwatered.

Hellriegel (1895) also made observations on the factors influencing nodule production. He says: "The best development and largest number of the tubercles are attained in soils quite free of nitrogen, and if the soil contains very much nitrogen the formation of tubercles may be altogether suppressed even when the fungus necessary to produce them is present."

The effect of calcium oxid on nodule formation has been observed by Salfeld-Lingen (1900). In 1893 he applied 2000 kilograms of quicklime per hectare to light sandy soil and in 1894 observed its effect on nodule production. Where the lime was applied the pea plants were dark green and the roots rich in nodules, while on the check field the plants were yellow and no nodules were found.

Marchal (1901) determined the concentrations of various substances that prevented nodule formation in Sachs' solution, which presumably had the following composition:

| | |
|---|---|
| Potassium nitrate | 1.0 gram |
| Sodium chloride | 0.5 gram |
| Calcium phosphate | 0.5 gram |
| Calcium sulfate | 0.5 gram |
| Magnesium sulfate | 0.5 gram |
| Water | 1000 cubic centimeters |

His conclusions may be briefly summarized as follows: The alkaline nitrates in concentrations of 1 to 10,000 and ammonium salts in concentrations of 1 to 2000 prevent nodule formation. The salts of potassium and of sodium behave similarly in concentrations of 1 to 200 and 1 to 300, respectively. The salts of calcium and of magnesium favor the production of nodules on the roots of the plants. Phosphoric acid has a stimulating effect. Marchal believes that the osmotic relationships are concerned with the inhibition of nodule formation.

Malpeaux (1901) also has observed the action of nitrates on nodule production of lupines. The addition of nitrate of soda diminished nodule formation and in a certain concentration prevented nodule development.

Laurent (1901) has contributed more extensively to this subject. He employed a variety of peas, and used small soil plats which were fertilized with a number of substances in excess of what the plants would use. He found: (1) that plants from the ammonium sulfate plat had only a few nodules, these being distributed over the lateral roots; (2) that plants from the plat receiving potassium salts were abundantly provided with nodules, which were massed together on the main root; (3) that with superphosphate of lime present, plants showed abundant formation of nodules, which were massed together on the principal roots; (4) that lime alone stimulated nodule production in great masses; (5) that sodium chloride stimulated the formation of numerous small nodules.

Wohltmann (1902) grew Canada field peas in a variety of soils and with a number of different treatments. He concluded that ammonium nitrate, if applied in liberal quantities, may reduce the number of nodules. Plants grown in one soil, no matter what treatment was applied, produced no nodules. Wohltmann points out that these results are significant with respect to the choice of fertilizers in relation to their effect on the bacteria in the soil.

Moore (1905) states that the alkaline nitrates in the proportion of 1 to 10,000 are sufficient to prevent the formation of nodules, and shows by photographs that soybeans grown in a poor sandy soil and in a poor

clay soil have a greater supply of nodules than plants grown in a rich nitrogenous soil. He states also that fully as striking differences might be shown in a soil in which the moisture or the acidity or the air supply varies, and that the application of calcium or magnesium will act differently on nodule production depending on whether the plant grows under acid or under alkaline conditions.

Donnan (1912) observed that lucern plats which received lime as a fertilizer in connection with inoculation experiments showed a very great increase in the number of plants with nodules and also an increase in the number of nodules per plant; where no bacterial culture was used for lucern, however, the presence of lime caused practically no difference in nodule formation. Also, "complete manure did not, on the total throughout, increase the number of nodules, although an increased growth of both stem and root was caused by its application."

Eichinger (1913), in testing the value of fertilizers in connection with inoculation experiments for soybeans, cowpeas, and other crops, observed that superphosphate increased the production of nodules, whereas sodium nitrate inhibited nodule formation.

Prucha (1915), in experiments with Canada field pea, found that certain substances when added to water culture or soil culture materially influenced nodule formation. The nitrates of calcium and potassium, as well as the chlorides of ammonium, calcium, and iron, when added to the soil in certain quantities inhibited nodule formation. This was true also of saccharose and of Witte's peptone. On the other hand, potassium dihydrogen phosphate, magnesium sulfate, potassium hydroxide, calcium phosphate, calcium sulfate, tannic acid, and starch, exerted a beneficial influence on nodule development. In water culture the presence of nitrogen as calcium nitrate or as potassium nitrate depressed nodule formation. Prucha determined also the influence on nodule formation of aëration, of some nutrient solutions, and of potassium nitrate, in light as compared with darkness. Aëration had no influence as measured in this work. A smaller number of nodules developed in light than in darkness.

In concluding a review of the literature it may be said that there are many factors which have more or less bearing on nodule formation and it is doubtful whether any one of the investigators has properly controlled all of them. This is especially so with all field tests, for here it is impossible to control the moisture relations and the concentration of the soil solution, both of which are known to bear intimate relations to nodule formation. Those who have worked with water culture have more nearly approached an ideal condition, at least a condition in which they have controlled many of these known influences. There are undoubtedly many factors,

such as temperature, age of the root, age of the entire plant, its condition of thrift, and the like, which bear some relation to nodule formation and which have been given little or no consideration in work of this kind. Whether these unknown influences are potent enough to materially alter the results already obtained can be told only as more light is thrown on this subject by experimental work.

## METHODS OF THE PRESENT INVESTIGATION

A somewhat definite outline of the methods employed in the experiments reported in this bulletin is necessary and is here given. The experiments made, unless otherwise indicated, were conducted in accordance with these methods.

### *The medium*

The medium employed for the isolation and cultivation of *Bacillus radicicola* of soybean is a modification of Ashby's medium and was prepared according to the following formula:

| | |
|---|---|
| Dibasic potassium phosphate | 0.2 gram |
| Magnesium sulfate | 0.2 gram |
| Sodium chloride | 0.2 gram |
| Calcium carbonate | 0.2 gram |
| Calcium sulfate | 0.2 gram |
| Agar | 15.0 grams |
| Saccharose | 20.0 grams |
| Tap water to make | 1000 cubic centimeters |

Test tubes were prepared containing from 6 to 10 cubic centimeters of this solution, and in each tube a crushed soybean was placed.

### *Sterilization of media and utensils*

Strict precautions were observed in securing sterile material wherever it was necessary. All the media used for pure cultures, when in small volume, were sterilized in the autoclave for fifteen minutes at 15 pounds pressure. Media in larger volumes were left in the autoclave for a longer period of time. In the case of soil cultures the containers and the soil were sterilized for two hours at a pressure of 15 pounds.

### *Sterilization of seed*

For certain experiments it was desirable to have seed free not only of *B. radicicola* but also of all other organisms. To this end, seeds were treated for three hours in a solution of calcium hypochlorite (Wilson, 1915) containing approximately 2 per cent of chlorine. After this treatment they were transferred to the culture containers.

*The organism*

The original culture for investigation was made from a stock culture which had been kept in the laboratory for two years or more, the exact date of isolation not being known. Transfers had been repeatedly made during this period, employing the medium previously described. This stock culture had also been repeatedly tested for its ability to produce nodules, with positive results. It was the culture from which hundreds of pure cultures have been prepared for distribution to farmers of the State.

*Culture methods for plants*

Both soil and water cultures were employed. The soil cultures were prepared by placing the equivalent of 208 grams of dry soil in glass tumblers

FIG. 80. METHOD OF ARRANGING SOIL CULTURES

having a capacity of approximately 250 cubic centimeters. The moisture was controlled after the seeds were planted. For the water cultures tumblers were used in some experiments, and in others glass cylinders of about 4700 cubic centimeters capacity were substituted. The solutions for these containers were prepared from stock solutions, placed directly in the container, and covered immediately with paraffin paper, which was held firmly to the container by a string or a rubber band. Plants grown in these culture solutions were not under strictly sterile conditions, yet in no case did the control cultures show nodule production.

The soil cultures for testing the vitality of *B. radicicola* under various cultural conditions were prepared in glass tumblers (page 400). Each tumbler was filled to within two centimeters of the top with air-dry, sandy soil, covered with heavy wrapping paper, and sterilized as previously

described. These tumblers were kept covered until planting time, when the covers were removed only long enough for planting the seeds and watering. The cultures were then transferred to the greenhouse and placed in an especially constructed culture room (fig. 80). Here they remained covered until the plants were in need of light for growth. This required five days out of the fourteen which were allowed for nodule development. The remaining nine days were not sufficient to permit disturbing conditions.

*Inoculation of plants*

The same methods were used to inoculate both soil and water cultures. The cultures were inoculated twenty-four hours after planting, the method being as follows: A small quantity of water was added to a fresh growth of the organism on an agar slope. The growth was then loosened by means of a platinum loop, and added to 100 cubic centimeters of sterile water. In order to secure inoculation a definite amount of this infusion, usually 5 cubic centimeters, was added to each culture. Platings of this infusion showed as many as 10,000,000 germs per cubic centimeter.

*Examination of roots for nodules*

Repeated trials showed that nodules macroscopically visible will develop under favorable conditions in fourteen days. However, in order to show the influence of certain factors on nodule formation and to eliminate all doubt as to the results obtained, and also to make the results more decisive, the roots were not examined in many of the experiments until after a considerably longer time had elapsed. In all cases the exact time is indicated. In determining the presence or the absence of nodules and the number produced in soil culture, the roots were first freed of soil by gently washing them and the counts were then made. For purposes of comparison the nodules are arbitrarily classed under groups, the classification being based on size and distribution; those 1 millimeter or more in diameter are designated as large, and those less than 1 millimeter in diameter are designated as small.

*The culture room*

In order to prevent contamination of the plant cultures growing in tumblers with *B. radicicola*, and to reduce evaporation from the free surfaces of the exposed containers, an especially constructed culture room was provided in the greenhouse. To secure ventilation certain panes of glass were replaced by frames fitted with layers of cotton held in place by cheesecloth. In winter these frames could be removed when necessary and replaced by frames containing glass. Cracks in the walls between the glass parts were filled with plaster of paris. The floor and the benches

were covered with cinders so as to reduce danger of contamination thru dust. Before using the room the walls and wooden parts were always thoroly washed.

*Watering the plants*

The water cultures had enough of the solution to supply their needs during the experimental period. With the soil cultures it was different. In order to maintain an optimum and somewhat definite moisture condition for growth it was necessary to water the plants every day. In all experiments, unless otherwise stated, distilled water was used. This was necessary in order to avoid the addition of substances that might have an influence on nodule formation.

*Growing plants under sterile conditions*

In determining the causal organism of nodule production, nodules must be produced in the presence of only the one organism in question. To accomplish this, it is necessary to grow the plant and the organism to the exclusion of all other forms of life. For this purpose a long-necked, flat-bottomed, four-liter flask was used in these experiments. In this flask was placed 1800 grams of sandy soil with a moisture content of about 16 per cent. The flask was plugged with cotton, thru which passed a glass tube with a bore large enough to allow the passage of a soybean. This tube also was plugged with cotton. At the time of seed planting the upper end of the glass tube was heated, the cotton was removed, and the seed was taken from the disinfecting solution and put into the flask thru the tube. The tube served also in placing and burying the seed, as well as providing an inlet for introducing the suspension of bacteria for inoculation.

*Obtaining plantlets for water cultures*

Soybeans (of the variety Hollybrook) were placed in a vessel, soaked in running water for three or four hours, and germinated between filter paper. Within a week germination was usually sufficiently advanced so that when brought into the light the cotyledons would turn green. At this stage they were ready for use, and the hypocotyl could be inserted into the culture solution thru a hole in the covering of the vessel.

*Soil type used*

In order to have as uniform conditions as possible for experiments of this kind, a well-defined New York State soil type was selected. This type was chosen mainly because of its accessibility and its agricultural importance. Owing to its water-holding capacity, the Volusia silt loam soil is adapted mostly to annual plants, especially the annual legumes,

the biennial plants failing because of frequent heaving of the soil in winter or because of the deficiency in the soil of some particular element, probably calcium.

The soil for the experimental work was procured from a farm in the vicinity of Ithaca. This soil had not been cultivated for at least twenty years, if at all, for it was taken from a strip dividing two fields. Since this soil had not been cultivated, it is probable that no kind of fertilizing material had ever been added to it.

On October 16, 1913, the top six or seven inches of soil from this field was brought to the college greenhouse and thoroly mixed after screening. The screenings were discarded. This mixed soil was placed in boxes, each box being filled and kept thoroly tight so that only a slight amount of moisture was lost. As soil was needed it was taken from the box, a moisture determination was made, and then enough soil was weighed into tumblers to equal 208 grams of dry soil. The chemical to be added to the soil, if soluble, was dissolved in just enough water so that when this was added to the soil it would give a moisture content of approximately 35 per cent of the dry weight of the soil. This percentage of moisture was used in all the soil tests. If the substance to be added was not soluble it was mixed with the soil, and the amount of water necessary to make a moisture content of 35 per cent was then added. This water content was maintained constant by daily weighings and addition of the necessary quantity of water.

### *Necessity of artificial inoculation*

It is a common observation that leguminous plants which are grown on a soil for the first time may be void of nodules. Therefore, in order

TABLE 1. RESULTS OF EXPERIMENT TO DETERMINE THE NECESSITY OF ARTIFICIAL INOCULATION

| Species tested | Nodules |
|---|---|
| Vigna unguiculata (cowpea) | None |
| Soja max (soybean) | None |
| Trifolium alexandrinum | None |
| Medicago lupulina | None |
| Medicago sativa | None |
| Medicago officinalis | None |
| Medicago falcata | None |
| Medicago media | None |
| Melilotus alba | None |
| Melilotus indica | None |
| Trifolium pratense | Present |
| Vicia villosa | None |
| Vicia sativa | None |
| Vicia dasycarpa | None |
| Vicia angustifolia | None |

to determine whether it was necessary to inoculate the soil cultures with *B. radicicola*, tumblers were prepared with the Volusia silt loam soil in them and planted with a number of different legume seeds, including soybean. The tumblers were watered daily but no effort was made to maintain a definite amount of water in the soil. The results are presented in table 1.

Of the fifteen leguminous plants grown in this soil, nodules were produced only in *Trifolium pratense*. It is therefore necessary, for nodule production, to inoculate each soybean culture with *B. radicicola* of soybean.

### *Normal production of nodules*

How widely the number of nodules per plant will vary under uniform conditions has not been determined. The data that have been obtained by other investigators along this line are so varied that no conclusions can be drawn. This is probably due to a lack of controlling the many factors influencing nodule production.

In order to obtain data that would be of value in drawing conclusions from the results that follow, a test was made to determine what the normal production of nodules may be in a given soil. A series of tumblers were prepared with soil and planted with soybeans, which were selected for uniformity of shape and size. The various portions of the soil were equally inoculated with the soybean culture and were kept at a definite moisture content by daily weighings. The data from one hundred plants are given in table 2:

TABLE 2. NORMAL PRODUCTION OF NODULES IN SOIL CULTURE
(Duration of experiment, 14 days)

| Number of plants examined | Number of nodules | Number of nodules per plant | Number of nodules per plant, average of 50 plants | Number of nodules per plant, average of 100 plants |
|---|---|---|---|---|
| 25 | 89 | 3.56 | 3.70 | |
| 25 | 86 | 3.44 | 3.50 | |
| 25 | 88 | 3.52 | 3.54 | |
| 25 | 96 | 3.84 | 3.64 | 3.59 |
| | | | 3.68 | |
| | | | 3.48 | |
| Average | ..... | ..... | 3.62 | ..... |
| Variation | ..... | ..... | ±0.12 | ..... |

These data show that the normal production of nodules per plant under the conditions of this experiment is between three and four. This

is true no matter whether twenty-five, fifty, or one hundred plants are considered. Figured to a basis of one hundred plants, the normal variation is ± 12. That this number is quite reliable is shown by the results from one hundred and twenty-five plants which were grown as check plants in subsequently reported experiments. These one hundred and twenty-five plants produced 350 nodules per 100 plants and the theoretical number would be 359. Therefore, in the experimental data which follow, the number of nodules per 100 plants which fluctuates more than ± 12 from that shown by the check plants may be considered as due to the condition under which the plants were grown.

## IDENTIFICATION OF BACILLUS RADICICOLA OF SOYBEAN

No detailed description of the soybean organism has as yet appeared, tho occasional incidental references are found concerning it. This is undoubtedly due in part to the difficulties encountered in cultivating the organism, in determining its motility, and in staining the flagella.

The source of the culture used in this work has been described and its ability to produce nodules is known. In order, however, to prove definitely that the culture employed was of the soybean organism and not a mixture, the following procedure was adopted for the reisolation of the organism preparatory to a study of the physiological and morphological characters:

1. Soybean plants were grown under sterile conditions and the roots inoculated with the culture.

2. When nodules were developed a new isolation was made from the nodule.

3. The original culture, and the cultures isolated from the nodule produced under sterile conditions, were then studied in the laboratory with respect to their morphological and physiological characters. Their group number was determined according to the descriptive chart of the Society of American Bacteriologists (1907).

4. All the cultures were again tested under sterile conditions for their ability to effect inoculation.

### *Details of identification methods*

For growing plants under sterile conditions the methods previously described were followed. The plants were prepared on December 27, 1913, and were inoculated on the following day with the organism in question. The flasks were then placed in the greenhouse, where they remained until February 28, 1914. At that time they were brought to the laboratory and the roots were examined for nodules. At the same time the soil from the flasks was examined for bacteria by planting it in both peptone beef-extract agar and in the modified Ashby's medium.

There were abundant nodules on the roots of the plants that were grown only in the presence of the organism in question. In seven days the plantings in the above-mentioned media were examined. Those made in peptone beef-extract agar showed no growth at all, while those made in the modified Ashby's medium showed a pure culture of some organism which resembled in every way, as judged from plantings, the organism that was introduced into the soil. The check plant showed no nodule development, and plantings of the soil showed no bacterial growth, on either medium used.

Two of the nodules produced were used for reisolation of the cultures. The pieces of root with nodules were first washed in mercuric chloride solution 1–1000 for two minutes, and then rinsed several times in sterilized water. Plantings of the last water used showed no bacterial growth. Plantings of the interior of these nodules developed a pure culture of some organism resembling in every way the culture introduced into the soil. From one of the plantings a colony was selected which was in all probability produced from one germ. A transfer was made from this colony to an agar slope. The same method was followed in obtaining another culture from a second nodule. These cultures were replanted along with the original culture, and their purity was determined.

A delay of several months occurred before the cultures were compared and their morphological and physiological characters determined. In June their detailed features were determined and a brief characterization was made. The methods for staining the flagella were as follows:

The organism was stained according to Pitfield's method as modified by Muir and Ritchie (1907). The mordant used was made according to the following formula:

| | Cubic centimeters |
|---|---|
| Tannic acid, 10 per cent water solution filtered | 10 |
| Corrosive sublimate, saturated watery solution | 5 |
| Alum, saturated watery solution | 5 |
| Carbol fuchsin | 5 |

These substances were mixed thoroly and filtered before using. The stain consisted of the following:

| | Cubic centimeters |
|---|---|
| Alum, saturated watery solution | 10 |
| Gentian violet, saturated alcoholic solution | 2 |

Films were prepared from cultures on agar which were grown at room temperature for from one day to seven days. A very small portion of the growth was taken on the loop of a platinum needle and carefully

transferred to water on a cover glass. This was allowed to dry in the open room, was fixed by passing thru a flame, and was covered with the mordant. The cover glass was covered with as much of the mordant as it would hold, and was heated gently over the flame. It was allowed to steam for about two minutes and was then washed well in running water. When it was thoroly dry the stain was applied, and the cover glass was heated as before for about a minute and then thoroly washed in water.

Examination of preparations made in this way showed that the flagella were peritrichous, the highest number found being four.

A comparison of these three cultures show them to be identical in every particular. They do not form spores, they are facultative anaërobes, and they do not liquefy gelatin either at room temperature (from 20° to 26° C.) or in the ice box (from 10° to 18° C.). No gas nor acid is produced from dextrose, lactose, or saccharose, and nitrates are not reduced. The cultures are colorless on agar slopes altho they are somewhat glistening in character. After two weeks they are slightly brownish, both on agar slopes and on potato cultures. The organism stains readily with most stains, and gives slightly different measurements under different cultural conditions. These measurements may range from 0.6 $\mu$ to 1.2 $\mu$ broad by from 0.8 $\mu$ to 3 $\mu$ long. Occasionally X and Y forms are observed, this also depending on the culture medium.

The last step in this determination of the causal organism was to retest all cultures as to their ability to produce nodules. On July 3, 1914, flasks were prepared and sterilized soybeans were planted in them. Three days later transfers from the original cultures, which gave identical characters, were injected into the soil within the flasks, which were then placed in the greenhouse. On July 15 the roots of these plants were examined for nodules.

The original culture and the two cultures which had been isolated from nodules grown under sterile conditions effected inoculation. The roots of the check plants were entirely void of any structures resembling nodules.

*Conclusions*

It would seem from these data that the organism in question was the one responsible for nodule formation, and that according to its detailed features it is a Bacillus, the flagella being attached to any part of the body. The highest number found in any preparation was four. Its group number, therefore, according to the classification of Migula (1900) and as determined by the descriptive chart of the Society of American Bacteriologists (1907), is B.222.3332033.

*Discussion*

The results of these findings are in accord with the findings of those who hold that the flagella of the organism are peritrichous and not polar. Its group number is slightly different from that given to *B. radicicola* of Canada field pea by Prucha (1915). He finds that this organism produces acid without gas from dextrose and saccharose. Under the same conditions the culture from soybean grew so little, if at all, that no change was recorded by the method used.

## INFLUENCE OF CERTAIN CHEMICAL SUBSTANCES AND OF SOIL MOISTURE ON NODULE FORMATION

As pointed out in the review of literature (page 369), various phases of the subject relating to the factors influencing the formation of nodules have been touched on from time to time. As yet no comprehensive work has appeared. In the investigations herewith reported, the writer has endeavored to determine the influence on nodule formation of a large number of substances both nutrient and non-nutrient, as well as of carbohydrates and other substances, in soil cultures and in water cultures.

*Soil cultures. Volusia silt loam*

*Nitrates*

On February 21, 1914, tumblers were filled as has been described, and a number of different nitrates applied to the soil. For each nitrate there were ten tumblers, each containing 208 grams of soil. The rate of application of the different nitrates to this soil was not the same in all cases, because of the variation in toxic effect of the different salts on the plant. The application of the salt is, however, not an arbitrary one, for in a previous experiment of the same nature the optimum concentration for each nitrate was determined for plant growth. The amount of salt added, however, is in all cases in excess of what would be used in field practice; yet the results

FIG. 81. CHECK PLANTS

are suggestive of what might be expected from the use of nitrates. The data are given in tables 3 and 4.

It is seen from table 3 that as a rule the application of nitrates greatly reduces the number of nodules formed. Of the eighteen nitrates used, only two permitted a total number of nodules greater than 165 per 100 plants. Not only are the numbers and the sizes reduced, but also their location is different. With the check plants a greater proportion of the nodules are on the main roots than is the case with those plants that have been grown under the influence of the nitrates.

In a preliminary experiment in which the plants were examined fifty days after planting, certain nitrates completely checked the appearance of nodules. That this checking of nodule formation is not due to the injurious effect of the nitrate on plant growth is seen from table 4.

In this experiment the nitrates were applied

FIG. 82. ROOT OF CHECK PLANT, NATURAL SIZE, SHOWING SMALL NODULES

TABLE 3. INFLUENCE OF NITRATES ON NODULE FORMATION
(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Calcium nitrate | 0.1 | 63 | 0 | 5 | 0 | 80 | 85 | 135 |
| Potassium nitrate | 0.1 | 44 | 0 | 6 | 0 | 63 | 69 | 157 |
| Ammonium nitrate | 0.1 | 49 | 0 | 0 | 0 | 4 | 4 | 8 |
| Magnesium nitrate | 0.1 | 64 | 0 | 3 | 0 | 45 | 48 | 75 |
| Sodium nitrate | 0.1 | 62 | 0 | 8 | 0 | 48 | 56 | 90 |
| Aluminium nitrate | 0.1 | 60 | 0 | 26 | 0 | 188 | 214 | 357 |
| Barium nitrate | 0.1 | 56 | 0 | 13 | 0 | 36 | 49 | 88 |
| Ferric nitrate | 0.1 | 57 | 0 | 8 | 0 | 38 | 46 | 81 |
| Lead nitrate | 0.1 | 53 | 0 | 4 | 0 | 8 | 12 | 23 |
| Cadmium nitrate | 0.02 | 57 | 0 | 0 | 0 | 12 | 12 | 21 |
| Cerium nitrate | 0.1 | 58 | 0 | 13 | 0 | 46 | 59 | 102 |
| Strontium nitrate | 0.1 | 59 | 0 | 0 | 0 | 27 | 27 | 46 |
| Lithium nitrate | 0.1 | 71 | 0 | 6 | 0 | 11 | 17 | 24 |
| Zinc nitrate | 0.05 | 59 | 0 | 10 | 0 | 69 | 79 | 134 |
| Uranium nitrate | 0.1 | 65 | 2 | 20 | 0 | 47 | 69 | 106 |
| Nickel nitrate | 0.02 | 62 | 0 | 30 | 0 | 72 | 102 | 165 |
| Mercuric nitrate | 0.1 | 62 | 0 | 28 | 0 | 44 | 72 | 116 |
| Nitric acid | 0.05 | 60 | 8 | 184 | 0 | 15 | 207 | 345 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

in a series of concentrations ranging from what would seem to be a heavy application to a very light one. The test was run in duplicate. With these applications it was possible to tell what concentrations are effective in checking nodule formation, and what ones produce injury to the plants. The former was determined by an absence of nodules, and the latter by an optical comparison of the plants with checks grown under the same condition. The injurious action was usually manifest by difference in root growth, tho sometimes by browning of leaves.

TABLE 4. SHOWING AMOUNT OF NITRATE WHICH INHIBITS NODULE FORMATION, AND AMOUNT WHICH CAUSES INJURY TO PLANTS

(In 208 grams of dry soil. Duration of experiment, 50 days)

| Substance used | Amount necessary to inhibit nodule formation (grams) | Amount necessary to cause injury to plant* (grams) |
|---|---|---|
| Lithium nitrate | 0.2 | 1.0 |
| Cadmium nitrate | 0.1 | 0.1 |
| Sodium nitrate | 0.1 | 0.5 |
| Calcium nitrate | 0.2 | 1.0 |
| Uranium nitrate | 0.1 | 1.0 |
| Strontium nitrate | 0.2 | 0.5 |
| Zinc nitrate | 0.1 | 0.5 |
| Ferrous nitrate | 0.1 | 0.5 |
| Nickel nitrate | 0.05 | 0.1 |
| Ammonium nitrate | 0.1 | 0.5 |
| Mercuric nitrate | 0.1 | 1.0 |
| Cæsium nitrate | 0.2 | 0.5 |
| Silver nitrate | 0.05 | 0.05 |
| Potassium nitrate | 0.05 | 0.5 |
| Magnesium nitrate | 0.5 | 0.5 |
| Lead nitrate | 0.2 | 0.5 |
| Barium nitrate | 0.2 | 0.5 |

* The injury to plants was determined by comparing them with check plants grown under similar conditions.

This summary brings out very forcibly that considerably less quantities of nitrates than are necessary to produce injury to the plants may completely inhibit the formation of nodules. This was true in fourteen out of seventeen cases. In the cases of the nitrates of cadmium, silver, and magnesium, the nodule formation stopped with those applied concentrations that injured the plants.

*Chlorides*

The influence of chlorides on the formation of nodules was tested simultaneously with that of the nitrates, and the same check plants were

used in this and the following experiments. Here, as with the nitrates, the application of the chloride is somewhat higher than would usually be applied, but such quantities are often found in the soil. The results are given in table 5:

TABLE 5. INFLUENCE OF CHLORIDES ON NODULE FORMATION

(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Potassium chloride | 0.05 | 58 | 19 | 40 | 3 | 314 | 376 | 648 |
| Sodium chloride | 0.05 | 40 | 3 | 40 | 10 | 181 | 234 | 585 |
| Magnesium chloride | 0.05 | 38 | 0 | 9 | 20 | 337 | 366 | 963 |
| Calcium chloride | 0.05 | 50 | 3 | 19 | 6 | 492 | 520 | 1,040 |
| Cæsium chloride | 0.05 | 55 | 9 | 70 | 11 | 405 | 495 | 900 |
| Bismuth chloride | 0.05 | 50 | 0 | 12 | 7 | 332 | 351 | 702 |
| Rubidium chloride | 0.05 | 52 | 4 | 42 | 0 | 255 | 301 | 579 |
| Lithium chloride* | 0.05 | 50 | 1 | 25 | 1 | 74 | 101 | 202 |
| Cupric chloride | 0.05 | 41 | 14 | 28 | 5 | 150 | 197 | 480 |
| Aluminium chloride | 0.05 | 53 | 2 | 9 | 29 | 298 | 338 | 638 |
| Lead chloride | 0.05 | 45 | 1 | 27 | 8 | 207 | 243 | 540 |
| Ammonium chloride | 0.05 | 39 | 0 | 19 | 0 | 29 | 48 | 123 |
| Manganese chloride | 0.05 | 47 | 0 | 72 | 0 | 334 | 406 | 864 |
| Cobalt chloride* | 0.05 | 38 | 0 | 0 | 0 | 37 | 37 | 97 |
| Strontium chloride | 0.05 | 53 | 4 | 54 | 0 | 403 | 461 | 870 |
| Ferrous chloride | 0.05 | 63 | 10 | 35 | 0 | 326 | 371 | 589 |
| Ferric chloride | 0.05 | 54 | 1 | 60 | 0 | 404 | 465 | 861 |
| Nickel chloride* | 0.05 | 33 | 1 | 0 | 0 | 56 | 57 | 173 |
| Zinc chloride | 0.05 | 40 | 1 | 27 | 0 | 123 | 151 | 378 |
| Antimony trichloride | 0.05 | 53 | 7 | 2 | 15 | 161 | 185 | 349 |
| Barium chloride | 0.05 | 52 | 0 | 10 | 0 | 328 | 338 | 650 |
| Hydrogen chloride | 0.05 | 54 | 84 | 227 | 0 | 15 | 326 | 604 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

* Application toxic.

It is evident from table 5 that the chlorides have a decided stimulating effect on the formation of nodules. In seventeen cases out of the twenty-two in which the chlorides were applied, the number of nodules was greater per 100 plants than when no chlorides were applied to the soil (fig. 82). In three of the five cases in which the nodules were less than in the case of the check plants, the applications of the chlorides were toxic to the plants. In a preliminary experiment in which these three chlorides were applied in amounts not toxic, a stimulating effect was noted. With ammonium chloride there seems to be an inhibitory effect. This compound will be given further consideration later (table 8, page 390).

FIG. 83. PLANTS GROWN WITH SODIUM CHLORIDE

From left to right, plants grown with 0.2, 0.1, 0.05, 0.02, and 0.01 gram of sodium chloride, respectively, in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

FIG. 84. PLANTS GROWN WITH STRONTIUM CHLORIDE

From left to right, plants grown with 0.2, 0.1, 0.05, 0.02, and 0.01 gram of strontium chloride, respectively, in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

FIG. 85. ROOT OF PLANT GROWN WITH 0.01 GRAM OF POTASSIUM CHLORIDE. NATURAL SIZE

Plant grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

In the case of some of these chlorides, and possibly with a majority of them, the high results obtained may be due in part to the presence of the base with which the chloride is combined. The results with calcium chloride would fall into this class. However, the consistently beneficial results with nearly all the chlorides, and the beneficial effect of hydrochloric acid in which there is no base, would tend to discredit the view that the base is a factor.

In a preliminary experiment in which the chlorides were applied in quantities sufficient to injure plant growth, they did not completely inhibit nodule formation. When 0.2 gram of copper chloride was applied, nodules were produced, while plants grown in the presence of 0.05 gram showed injury, which was evident by the appearance of brown spots on the roots. With cobalt chloride nodules were produced on the plant roots under applications of 0.1 gram, while the roots were injured by application of 0.05 gram per 208 grams of soil.

FIG. 86. ROOT OF PLANT GROWN WITH 0.01 GRAM OF SODIUM CHLORIDE. NATURAL SIZE

Plant grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

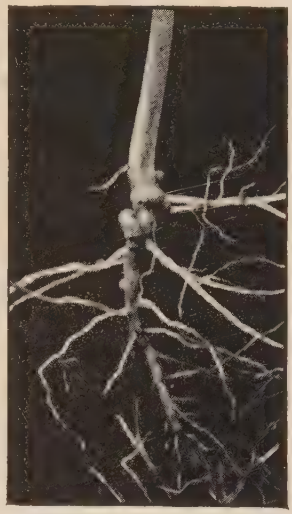

FIG. 87. ROOT OF PLANT GROWN WITH 0.01 GRAM OF STRONTIUM CHLORIDE. NATURAL SIZE

Plant grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

These results are quite in accord with those obtained by Laurent (1901), but are just the reverse of those obtained by Prucha (1915), who concluded that under the conditions of his experiments the chlorides of ammonium, potassium, and iron inhibit nodule formation in Canada field pea. These differences may be due to the use of a different host plant, but more probably they are due to differences in experimental procedure.

*Sulfates*

The influence of sulfates on the formation of nodules was tested in the greenhouse simultaneously with the study of the nitrates and the chlorides. The conditions for all three experiments were the same. The results with the sulfates are given in table 6:

TABLE 6. INFLUENCE OF SULFATES ON NODULE FORMATION
(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Sodium sulfate | 0.05 | 65 | 11 | 46 | 2 | 53 | 112 | 172 |
| Manganese sulfate | 0.05 | 59 | 22 | 68 | 0 | 151 | 241 | 408 |
| Magnesium sulfate | 0.05 | 58 | 7 | 21 | 1 | 32 | 61 | 105 |
| Calcium sulfate | 2.00 | 61 | 12 | 101 | 0 | 53 | 166 | 272 |
| Barium sulfate | 2.00 | 63 | 16 | 93 | 1 | 92 | 202 | 321 |
| Potassium sulfate | 0.05 | 58 | 8 | 67 | 0 | 134 | 209 | 360 |
| Zinc sulfate | 0.05 | 61 | 8 | 67 | 0 | 104 | 179 | 293 |
| Copper sulfate | 0.05 | 61 | 2 | 11 | 0 | 48 | 61 | 100 |
| Nickel sulfate* | 0.05 | 52 | 0 | 9 | 16 | 79 | 104 | 200 |
| Lithium sulfate | 0.05 | 68 | 5 | 21 | 4 | 103 | 133 | 196 |
| Aluminium sulfate | 0.05 | 51 | 9 | 51 | 0 | 37 | 97 | 190 |
| Strontium sulfate | 2.00 | 66 | 6 | 117 | 1 | 89 | 213 | 323 |
| Mercuric sulfate | 0.50 | 44 | 0 | 9 | 0 | 15 | 24 | 55 |
| Iron tersulfate† | 0.05 | 56 | 9 | 39 | 1 | 99 | 148 | 264 |
| Iron and ammonium sulfate | 0.05 | 65 | 0 | 47 | 0 | 132 | 179 | 275 |
| Ammonium sulfate | 0.05 | 60 | 1 | 12 | 0 | 26 | 39 | 65 |
| Calcium ethyl sulfate | 0.05 | 53 | 64 | 171 | 11 | 195 | 441 | 832 |
| Aluminium and ammonium sulfate | 0.05 | 62 | 19 | 47 | 0 | 273 | 339 | 547 |
| Chromium and potassium sulfate | 0.05 | 70 | 13 | 46 | 2 | 247 | 308 | 440 |
| Aluminium and potassium sulfate | 0.05 | 58 | 39 | 79 | 0 | 278 | 396 | 683 |
| Quinine sulfate | 0.05 | 61 | 32 | 57 | 0 | 273 | 362 | 593 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

* Roots show slight toxic effect from salt.
† This is iron *tersulfate*, not iron *persulfate*.

From these data it seems that the sulfates have a harmful effect on nodule formation. Of the twenty-one sulfates tested, fourteen show a reduction in nodule formation. Of these fourteen, mercuric sulfate and ammonium sulfate show the greatest depressing effect. Plants grown in the presence of 0.05 gram of potassium sulfate show 10 more

nodules per 100 plants than do the checks, which is well within the normal variation. Those grown under the influence of manganese sulfate show 58 more nodules per 100 plants than do the checks. It would hardly seem that this is due to normal variation, but rather to the apparent stimulation of root growth by manganese, thus making possible a greater chance for infection. With calcium ethyl sulfate it is probable that the calcium, and perhaps the ethyl part of the compound, may have a beneficial influence on nodule formation. A further discussion of the calcium compounds is given on page 391. The aluminium and ammonium sulfate and the chromium and potassium sulfate have a noticeable stimulating effect. This was noted also with aluminium when applied as a nitrate, in which case the number of nodules per 100 plants was equal to or greater than the number per 100 plants of the check (table 3). Quinine sulfate increased the number of nodules from 350 per 100 plants, as shown by the checks, to 593. Undoubtedly the last-named result was due to the presence of the alkaloidal radical. In a preliminary test with caffein and amygdalin a stimulating effect was noted.

FIG. 88. ROOT OF PLANT GROWN WITH 0.01 GRAM OF SODIUM SULFATE. NATURAL SIZE

Plant grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

FIG. 89. ROOT OF PLANT GROWN WITH 0.01 GRAM OF POTASSIUM SULFATE. NATURAL SIZE

Plant grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

*Phosphates*

The influence of phosphates on nodule formation was tested simultaneously with that of the nitrates. The results are shown in table 7.

The data show conclusively that under these conditions phosphates increase the formation of nodules. In every case in which phosphates were applied, save that of disodium phosphate, the number of nodules per 100 plants was greater than with the check. The lower results with the disodium phosphate would seem to be due to the addition of a greater quantity of sodium, since the application of the monosodium phosphate

more than doubled the number of nodules. The same relation holds with the di- and mono-potassium salts, altho the difference is not so marked.

TABLE 7. INFLUENCE OF PHOSPHATES ON NODULE FORMATION
(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Monosodium phosphate | 0.1 | 50 | 28 | 65 | 0 | 294 | 387 | 774 |
| Ferric phosphate | 0.1 | 64 | 38 | 70 | 3 | 260 | 371 | 580 |
| Phosphoric acid | 0.1 | 54 | 15 | 67 | 2 | 200 | 284 | 526 |
| Monopotassium phosphate | 0.1 | 63 | 7 | 159 | 0 | 237 | 403 | 640 |
| Dipotassium phosphate | 0.1 | 48 | 10 | 128 | 0 | 115 | 253 | 527 |
| Disodium phosphate | 0.1 | 54 | 8 | 96 | 0 | 54 | 158 | 293 |
| Ammonium phosphate | 0.1 | 56 | 7 | 165 | 5 | 75 | 252 | 450 |
| Magnesium phosphate | 0.5 | 57 | 35 | 237 | 0 | 91 | 363 | 637 |
| Tricalcium phosphate | 0.5 | 59 | 18 | 137 | 0 | 69 | 224 | 380 |
| Monocalcium phosphate | 0.1 | 57 | 10 | 213 | 0 | 115 | 338 | 593 |
| Dicalcium phosphate | 1.0 | 62 | 7 | 157 | 0 | 66 | 230 | 371 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

*Ammonia-containing or ammonia-producing compounds*

The test to determine the influence of ammonia-containing or ammonia-producing compounds on nodule formation was made simultaneously with that of the nitrates. The results are given in table 8:

TABLE 8. INFLUENCE OF AMMONIA-CONTAINING OR AMMONIA-PRODUCING COMPOUNDS ON NODULE FORMATION
(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Ammonium sulfate | 0.05 | 60 | 1 | 12 | 0 | 26 | 39 | 65 |
| Ammonium carbonate | 0.05 | 48 | 5 | 118 | 0 | 43 | 166 | 346 |
| Ammonium nitrate | 0.1 | 49 | 0 | 0 | 0 | 4 | 4 | 8 |
| Aluminium and ammonium sulfate | 0.05 | 62 | 19 | 47 | 0 | 273 | 339 | 547 |
| Ammonium chloride | 0.05 | 39 | 0 | 19 | 0 | 29 | 48 | 123 |
| Ammonium phosphate | 0.1 | 56 | 7 | 165 | 5 | 75 | 252 | 450 |
| Dried blood | 1.0 | 55 | 11 | 177 | 0 | 8 | 196 | 356 |
| Witte's peptone | 0.5 | 57 | 0 | 162 | 0 | 9 | 171 | 300 |
| Ammonium hydroxide | 0.05 | 50 | 16 | 130 | 0 | 3 | 149 | 298 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

From these data it is evident that certain ammonia-containing compounds materially reduce the formation of nodules. Ammonium nitrate under these conditions depressed nodule formation from 350 per 100 plants, as shown by the checks, to 8 per 100 plants. Ammonium sulfate reduced the number from 350 to 65 per 100 plants. Ammonium chloride, ammonium hydroxide, and Witte's peptone also were effective in reducing the number of nodules. In this test dried blood shows no effect; in a previous test 2 grams per 208 grams of soil completely inhibited nodule formation.

A comparison between treatments of ammonium hydroxide and of hydrogen chloride (table 5), and no treatment, shows that the ammonia reduced the number of nodules 52 per 100 plants below the check, while the hydrogen chloride increased the number 254 per 100 plants over the check. All plants were grown under the same conditions.

Not all the applied compounds that contained ammonia, however, depressed nodule formation. Among those that did not were aluminium and ammonium sulfate, and ammonium phosphate. This would indicate that there may be a neutralizing effect on nodule formation with two substances, one of which will stimulate and the other retard nodule formation. This is shown by the following comparison of the data obtained from the application of certain salts:

| | Quantity applied | Nodules per 100 plants |
|---|---|---|
| Ammonium chloride | 0.05 gram | 123 |
| Ammonium nitrate | 0.1 gram | 8 |
| Aluminium chloride | 0.05 gram | 638 |
| Check | ..... | 350 |

These data indicate that where the anion and the cation in the same compound reduce the formation of nodules, the number of nodules per 100 plants is considerably smaller than where either one of the ions is combined with an element that is stimulative in effect. This is shown by ammonium nitrate. Here both the anion and the cation reduce nodule formation, but when the nitrate is replaced by the chloride, which is stimulative in effect, the number of nodules per 100 plants approaches more nearly that of the checks. Other comparisons could be made between calcium oxid, calcium sulfate, calcium chloride, and calcium nitrate (table 9). It is believed that if the right concentrations of these substances were used, this neutralizing effect could be made more pronounced.

*Calcium-containing compounds*

A test of the lime requirement of this Volusia silt loam according to the Veitch method shows that about 1100 pounds of calcium oxid per

TABLE 9. INFLUENCE OF CALCIUM-CONTAINING COMPOUNDS ON NODULE FORMATION

(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Calcium oxid | 0.2 | 53 | 125 | 454 | 0 | 95 | 674 | 1,272 |
| Calcium hydrate | 1.0 | 61 | 13 | 249 | 0 | 205 | 467 | 766 |
| Calcium sulfate | 2.0 | 61 | 12 | 101 | 0 | 53 | 166 | 272 |
| Calcium chloride | 0.05 | 50 | 3 | 19 | 6 | 492 | 520 | 1,040 |
| Tricalcium phosphate | 0.5 | 59 | 18 | 137 | 0 | 69 | 224 | 380 |
| Calcium ethyl sulfate | 0.05 | 53 | 64 | 171 | 11 | 195 | 441 | 832 |
| Calcium nitrate | 0.1 | 63 | 0 | 5 | 0 | 80 | 85 | 135 |
| Calcium carbonate | 1.0 | 58 | 4 | 231 | 0 | 168 | 403 | 695 |
| Calcium saccharate | 0.7 | 56 | 113 | 213 | 2 | 353 | 681 | 1,216 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

8,000,000 pounds of soil is necessary to neutralize it. One would expect, therefore, that an application of this material would stimulate nodule formation. The results on nodule formation of such a treatment, along with those from some other calcium-containing compounds, are presented in table 9.

FIG. 90. ROOT OF PLANT GROWN WITH 0.2 GRAM OF CALCIUM OXID. NATURAL SIZE

Plants grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

The striking fact shown in this table is that certain calcium-containing compounds produce exceptionally favorable conditions for the development of nodules. First among these is calcium oxid (fig. 90). This compound gave the greatest stimulating effect of all the calcium-containing substances. It produced not only an increase in nodules, but also a change in their location on the root system. Of the 1272 nodules per 100 plants, 1092 were on the main root; and of these 1092, 236 were more than one millimeter in diameter. Calcium saccharate (fig. 91) is next in importance, giving 1216 nodules per 100 plants, and calcium chloride follows with 1040 nodules per 100 plants. As in the case of the oxid, these compounds have induced a greater number of nodules on the main root than have other non-calcium materials already discussed. The low results with calcium sulfate and calcium nitrate are

undoubtedly due to the neutralizing effect of the material with which the calcium is combined.

FIG. 91. ROOTS OF PLANTS GROWN WITH 0.75 GRAM OF CALCIUM SACCHARATE. NATURAL SIZE

Plants grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

*Carbonates*

Since carbonated water is nearly always found in the soil, it was thought well to ascertain what effect it might have on nodule formation. Data bearing on this point are presented in table 10:

TABLE 10. EFFECT OF CARBONATES ON NODULE FORMATION
(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Calcium carbonate | 1.0 | 58 | 4 | 231 | 0 | 168 | 403 | 695 |
| Barium carbonate | 1.0 | 61 | 7 | 225 | 0 | 97 | 329 | 539 |
| Ammonium carbonate | 0.05 | 48 | 5 | 118 | 0 | 43 | 166 | 346 |
| Sodium bicarbonate | 0.1 | 46 | 11 | 90 | 0 | 100 | 201 | 437 |
| Sodium carbonate | 0.1 | 48 | 6 | 132 | 0 | 64 | 202 | 421 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

It will be noted that in most cases the plants grown under the influence of the carbonates have more nodules per 100 plants than do the checks. However, if the influence of the alkaline radical with which the carbonate is combined were eliminated, this result would not be so apparent. From the data presented it cannot be said that the carbonate part of the compound has any influence on the formation of nodules.

*Various carbon compounds*

Various carbon compounds were applied to the soil and the effect on nodule formation was noted. It was necessary to apply the compound in rather excessive quantities in order to have it, or compounds resulting from its application, remain in the soil during the experimental period. Altho no tests were made to determine the length of time the compounds remained in the soil, it is believed that the applications were sufficient to permit them to continue in the soil during the infection period. The check plants in this case were those used in the experiments already described, and were grown at the same time with the plants to which the carbon compounds were applied. The results of these tests are given in table 11:

TABLE 11. INFLUENCE OF VARIOUS CARBON COMPOUNDS ON NODULE FORMATION

(In 208 grams of dry soil. Duration of experiment, 30 days)

| Substance used | Quantity applied (grams) | Number of plants | Number of nodules on main roots | | Number of nodules on other roots | | Total number of nodules | Number per 100 plants |
|---|---|---|---|---|---|---|---|---|
| | | | Large | Small | Large | Small | | |
| Glucose | 2.0 | 59 | 29 | 78 | 0 | 100 | 207 | 351 |
| Maltose | 2.0 | 50 | 58 | 122 | 0 | 105 | 285 | 570 |
| Fructose | 2.0 | 58 | 110 | 296 | 0 | 149 | 555 | 957 |
| Glycerin | 1.0 | 64 | 122 | 241 | 0 | 21 | 384 | 600 |
| Lactose | 2.0 | 53 | 128 | 185 | 0 | 13 | 326 | 615 |
| Saccharose | 2.0 | 51 | 72 | 137 | 0 | 33 | 242 | 475 |
| Starch | 4.0 | 45 | 149 | 263 | 0 | 134 | 546 | 1,213 |
| Oxalic acid | 0.5 | 47 | 100 | 143 | 0 | 92 | 335 | 713 |
| Lactic acid | 0.5 | 63 | 88 | 319 | 0 | 104 | 511 | 811 |
| Citric acid | 0.5 | 57 | 86 | 262 | 0 | 108 | 456 | 800 |
| Sodium tartrate | 0.02 | 52 | 59 | 100 | 0 | 158 | 317 | 610 |
| Potassium tartrate | 0.02 | 55 | 63 | 290 | 0 | 244 | 597 | 1,085 |
| Potassium citrate | 0.02 | 58 | 49 | 261 | 0 | 38 | 348 | 600 |
| Caffein | 0.03 | 50 | 46 | 302 | 0 | 53 | 401 | 802 |
| Calcium saccharate | 0.7 | 56 | 113 | 213 | 2 | 353 | 681 | 1,216 |
| Cumarin | 0.03 | 23 | 33 | 148 | 0 | 32 | 213 | 926 |
| Checks | ..... | 125 | 22 | 195 | 1 | 220 | 438 | 350 |

The influence of carbon compounds on the formation of nodules is readily seen from table 11. Without exception, the number of nodules per 100 plants was greater where the soil was treated with carbon compounds than was the case with the checks. The three compounds giving the best results were calcium saccharate, starch, and potassium tartrate (fig. 92), in the order given. These three produced more than 1000 nodules per 100 plants, while the checks produced only 350 nodules. It is important to note also the number of nodules on the main roots in these cases, and their size in comparison with those of the checks. Of the 1213 nodules per 100 plants produced under the influence of starch, 916 were on the main root; and of these 916, 331 were more than one millimeter in diameter — only 19 less than the total number of nodules found on the checks.

FIG. 92. ROOTS OF PLANTS GROWN WITH 0.02 GRAM OF POTASSIUM TARTRATE. NATURAL SIZE

Plants grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

FIG. 93. ROOTS OF PLANTS GROWN WITH 2 GRAMS OF FRUCTOSE. NATURAL SIZE

Plants grown in 208 grams of soil; moisture content, 35 per cent of dry weight of soil

Six other substances also more than doubled the production of nodules. These were fructose (fig. 93), with 957 nodules per 100 plants; oxalic acid, with 713; lactic acid, with 811; citric acid, with 800; caffein, with 802; and cumarin, with 926. These substances likewise produced a greater number of nodules on the main roots than on the secondary roots. The checks were about equally divided in this respect. These results indicate that the carbonaceous material was assimilated by the host plant and that this assimilation resulted in better nutrition — a factor which Vines believed to favor nodule production.

*Variation of soil moisture*

A test of the effect of moisture on nodule formation was conducted, using Volusia silt loam soil and adding to it the amount of distilled water necessary to give it the desired moisture content, which ranged from 25 to 75 per cent of the dry weight of the soil. Soil cultures were set up in the usual manner and five or six soybean plantlets were placed in each. The results are given in table 12:

TABLE 12. EFFECT OF MOISTURE ON THE FORMATION OF NODULES
(Soil cultures. Duration of experiment, 17 days)

| Per cent of moisture | Number of plants | Number of nodules | Number of nodules per 100 plants |
|---|---|---|---|
| 25 | 30 | 1 | 3 |
| 35 | 26 | 50 | 192 |
| 45 | 31 | 148 | 477 |
| 55 | 30 | 242 | 807 |
| 65* | 34 | 408 | 1,200 |
| 75* | 28 | 394 | 1,407 |

* With a moisture content of 65 and 75 per cent, free water stood above the soil in the containers.

The figures in table 12 show that as the moisture is increased the number of nodules is increased. This may be explained in several ways. The two most probable explanations are: (1) the greater the water content, the better is the opportunity for infection; or (2) the greater the water content, the more dilute is the soluble substance in the soil which may have an inhibitory effect on nodule formation. However, the presence of large amounts of moisture may result in a modification of the roots, the root hairs, or possibly the protoplasmic activities of the bacteria.

Moore (1905), in discussing this subject, says the effect of moisture is probably a physical one. Thru it the bacteria have a better opportunity to come in contact with the roots, and as a result a greater number of infections occur.

*Water cultures*

In this part of the work it was the intention to repeat in water culture, as far as possible, the work that had been done with soil. In order, therefore, to have no substances present other than such as would come from the container or from the seed, distilled water was used. Cylinders were employed, each containing 4700 cubic centimeters of water, to which was added the salt or salts to be tested. For each container ten plantlets were used. These were supported by paraffin paper covers, in such a

manner that their roots could extend thru openings in the covers into the water. Growth was permitted for from four to six weeks.

Since it was shown by Prucha (1915) that nodules were not developed on Canada field pea in the absence of any one of the essential elements except nitrogen when he used the salts of Pfeffer's nutrient solution in six liters of water, it was decided to repeat this experiment, using soybeans instead of the peas. Accordingly Pfeffer's solution was made as follows:

| | |
|---|---|
| Calcium nitrate | 4 grams |
| Potassium nitrate | 1 gram |
| Magnesium sulfate | 1 gram |
| Monopotassium phosphate | 1 gram |
| Potassium chloride | 0.5 gram |
| Ferric chloride | 0.005 gram |
| Water to make | 6 liters |

*Pfeffer's solution with salts in various combinations*

In making the tests in Pfeffer's solution with salts in various combinations, the full strength, and fifty-three of the possible sixty-three combinations, aside from the distilled water alone, were used. A list of these solutions is given on pages 402 and 403.

Careful examination of the roots was made after the treatment, and any tubercle-like swellings resembling nodules were sectioned and examined microscopically. Not a single nodule was observed on any plant root grown in any of the solutions. Many of the solutions employed permitted good growth, while some were so toxic that the plants scarcely remained alive.

Plants grown in tap water at the same time as those grown in Pfeffer's solution developed luxuriantly without the addition of any chemicals, and produced nodules abundantly in fifteen days. It was assumed that possibly the concentration of the solution was not favorable to nodule formation or that the distilled water was the inhibiting factor.

*Pfeffer's solution of various strengths*

The results of the preceding experiment led to a series of experiments in which the salts of Pfeffer's solution were again used in distilled water. The concentrations, however, differed from those in the preceding experiment. They were 8, 4, 3, 2, 1, 1/2, 1/4, 1/8, 1/16, and 1/32, of the concentration of the full-strength solution. These were prepared on September 3, and on October 7 the roots were examined for nodules.

In only two of these concentrations were nodules produced. In the double strength there was a single plant which had two nodules, both

below the water line. The other case was in the 1/4 concentration, in which there was one nodule slightly above the water line.

*Pfeffer's solution in the absence of nitrates*

As the concentrations used in the preceding experiment were not favorable to nodule formation, it was decided to repeat this work leaving out the nitrates. Accordingly the following strengths of Pfeffer's solution were used: 8, 4, 2, 1, 1/4, 1/8, 1/16, 1/32. Potassium chloride and calcium chloride in molecular equivalents were substituted for the nitrates.

The plants grew normally in all the concentrations except the highest. No nodules were found in any of the concentrations greater than 1/4 strength. In the 1/4-strength solution and in all the lower concentrations there were nodules produced, but in no case were they as numerous as in the tap-water cultures which had been prepared as a means of checking the time factor in nodule development.

*Magnesium sulfate in distilled water*.

The results obtained with Pfeffer's solution in various concentrations led to a test of these various salts individually in distilled water. The containers were 800-cubic-centimeter cylinders and were prepared as described on page 374. The amounts of magnesium sulfate in grams per liter of water were as follows: 2, 1, 1/2, 1/4, 1/8.

After standing for thirty-six days in the greenhouse the plants were examined for the presence of nodules. The plants had made a fair growth. The roots were short in the two strongest concentrations. No nodules were observed on any of the roots.

*Monopotassium phosphate*

This experiment is a parallel of the one with magnesium sulfate. The phosphate was used in concentrations of 2, 1, 1/2, 1/4, 1/8, grams per liter of water.

The plants were examined thirty-eight days after inoculation. Those in the concentrations greater than 1/4 gram per liter of water were dead. The other concentrations were not toxic to any noticeable extent. The plants were 12 inches tall. No nodules were evident.

*Distilled water shaken with carbon black*

The difficulties encountered in producing nodules in distilled water led to the treatment of the water with carbon black. This was done by shaking the distilled water for fifteen minutes with 2 grams of petroleum carbon black per liter of water. After filtering, the water was used in tumblers alone and in combination with certain chemical substances that

were known to have an influence on the formation of nodules when added to soil. The contents of the tumblers were inoculated with one cubic centimeter of a water suspension of *Bacillus radicicola*. In table 13 the results are compared with data obtained under identically the same conditions with plants grown in tap water:

TABLE 13. NODULE FORMATION IN DISTILLED WATER SHAKEN WITH CARBON BLACK, AS COMPARED WITH TAP WATER*

| Substance used | Concentration (given as molecular weight of compound in 1 liter of water) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Distilled water | | | | | | Tap water | | | | | |
| | 1/2500 | 1/3500 | 1/5000 | 1/10,000 | 1/50,000 | 1/100,000 | 1/2500 | 1/3500 | 1/5000 | 1/10,000 | 1/50,000 | 1/100,000 |
| Zinc nitrate | —t | —t | —t | —t | —t | + | —t | —t | —t | —t | —t | —t |
| Ammonium nitrate | — | — | — | + | + | + | — | — | — | — | + | — |
| Potassium nitrate | — | — | — | — | + | + | + | — | + | + | + | — |
| Calcium nitrate | + | + | + | — | + | + | + | + | + | + | + | + |
| Sodium nitrate | + | + | + | + | — | + | + | + | + | + | + | + |
| Strontium nitrate | —t | —t | — | + | + | + | — | — | + | + | + | + |
| Sodium chloride | + | — | + | + | + | + | — | + | + | + | + | + |
| Calcium chloride | — | + | + | + | + | + | — | + | + | + | + | + |
| Aluminium chloride | —t | —t | —t | +t | + | — | —t | —t | +t | + | + | + |
| Potassium chloride | — | — | — | + | — | + | — | + | + | + | + | + |
| Zinc chloride | —t | —t | —t | —t | + | — | —t | —t | —t | —t | + | + |
| Strontium chloride | —t | —t | — | + | + | — | + | + | + | + | + | + |
| Potassium sulfate | —t | —t | + | + | + | + | + | — | + | + | + | + |
| Ammonium sulfate | —t | —t | —t | —t | —t | —t | — | — | + | + | + | + |
| Aluminium sulfate | —t | —t | —t | —t | — | — | — | — | — | + | + | + |
| Strontium sulfate | —t | —t | —t | —t | —t | + | — | — | + | + | + | + |
| Sodium sulfate | —t | —t | + | + | + | — | — | — | + | + | + | + |
| Zinc sulfate | —t | —t | —t | —t | —t | + | —t | —t | —t | —t | —t | + |
| Checks | Only 2 nodules on 18 plants. | | | | | | + | + | + | + | + | + |

* + indicates nodules present; — indicates no nodules; "t" indicates concentration toxic to roots.

The results obtained with the distilled water shaken with carbon black without the addition of any substance are for the most part negative. Only two nodules were found on eighteen plants, and these were on one plant.

It should be noted that each nitrate changed the condition in the distilled water treated with carbon black to such an extent that nodules were produced, and also that as the concentration of the nitrate increased there was a tendency toward prevention of nodule production. Calcium

nitrate and sodium nitrate seem to be less inhibitory in their effect than the other nitrates used. In tap water the nitrates seem to produce about the same results.

Sodium and calcium chloride and sodium and potassium sulfate also appear to react favorably to nodule formation in the treated distilled water. With the sulfates this favorable action is present in as low a concentration as 1/50,000 molecular solution, and extends to concentrations that appear toxic to the plant roots. Their action is not so pronounced, however, as that of the chlorides.

## VITALITY OF BACILLUS RADICICOLA OF SOYBEAN UNDER CULTURAL CONDITIONS

In previously reported experiments it was shown that the formation of nodules was completely checked when a nitrate or a sulfate was applied to soil in sufficient quantities, even tho the application was far below the amount necessary to produce injury to the plants. The same effect was observed in water cultures. The question that immediately presented itself was, What was the cause for such inhibitory effects? Did these compounds destroy the organism, or was some other factor operating? In order to obtain data on this subject the soil and water cultures employed in the experiments already reported were used as a basis for further study.

### *In soil cultures*

In testing for the presence or the absence of the organism in those cases in which nodule formation was inhibited by the application of various substances, the following method was employed: At the time of examination of the roots for nodules, which was after fifty days, the culture vessels were tapped gently so that the soil and the roots would come out in a mass. This mass was broken open, and some of the soil (about 5 grams) from the center of the mass was taken on a sterile spatula. This soil was used as inoculating material for other sterilized tumblers which were partly filled with soil. These tumblers, after being inoculated, were planted with ten soybeans, watered, and left in the culture room. At the end of twenty days the roots were examined for nodules. The results, which are shown in table 14, were compared with checks grown under the same conditions.

The results indicate that the application of nitrates or sulfates to soil in quantities sufficient to prevent nodule formation does not destroy the organism, for in most cases when brought into favorable condition the organisms were, from all tests, as efficient as the original culture, or as those cultured organisms in the soil with the presence of a smaller amount of nitrate or sulfate. There are a few apparent exceptions to

this statement, these being the nitrates of mercury, uranium, and nickel, and the sulfate of mercury. Why such results should be obtained with these compounds is not known. It is possible that these substances in the soil have a greater toxic effect than the other compounds, or that they react with the soil in some way to form a germicide and thus destroy the legume organism.

TABLE 14. INFLUENCE OF VARIOUS SALTS IN SOIL ON THE VITALITY OF BACILLUS RADICICOLA*

(Presence of organism determined at the end of 50 days by its ability to produce nodules. Duration of test, 20 days)

| Substance used | Quantities applied in inhibiting concentrations, with results | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Quantity applied | Organism | Quantity applied | Organism | Quantity applied | Organism | Quantity applied | Organism | Quantity applied | Organism |
| Cerium nitrate | 1.0 | — | 0.5 | — | 0.2 | + | ..... | .... | ..... | ..... |
| Lead nitrate | 0.5 | — | 0 | .... | 0.2 | + | ..... | .... | ..... | ..... |
| Silver nitrate | 0 | .... | ..... | .... | ..... | .... | 0.05 | — | ..... | ..... |
| Mercuric nitrate | 1.0 | — | 0.5 | — | 0.2 | — | ..... | .... | ..... | ..... |
| Ammonium nitrate | ..... | .... | 0.5 | — | 0.1 | + | ..... | .... | ..... | ..... |
| Calcium nitrate | 1.0 | + | ..... | .... | ..... | .... | ..... | .... | ..... | ..... |
| Zinc nitrate | ..... | .... | 0.2 | + | 0.1 | + | ..... | .... | ..... | ..... |
| Uranium nitrate | 1.0 | — | 0.5 | — | 0.2 | — | 0.1 | — | ..... | ..... |
| Sodium nitrate | 1.0 | — | 0.5 | + | 0.2 | + | 0.1 | + | ..... | ..... |
| Ferrous nitrate | ..... | .... | ..... | .... | 0.2 | + | ..... | .... | ..... | ..... |
| Barium nitrate | ..... | .... | 0.5 | + | 0.2 | — | ..... | .... | ..... | ..... |
| Potassium nitrate | ..... | .... | 0.5 | + | 0.1 | + | 0.05 | + | ..... | ..... |
| Nickel nitrate | ..... | — | 0.2 | — | 0.1 | — | 0.05 | — | ..... | ..... |
| Magnesium nitrate | 1.0 | — | 0.5 | + | ..... | .... | ..... | .... | ..... | ..... |
| Strontium sulfate | 5.0 | + | 2.0 | + | 0.5 | — | ..... | .... | ..... | ..... |
| Copper sulfate | ..... | .... | 0.1 | — | ..... | .... | ..... | .... | ..... | ..... |
| Chromium and potassium sulfate | ..... | .... | 0.5 | — | 0.2 | — | 0.1 | — | 0.05 | + |
| Sodium sulfate | ..... | .... | 0.5 | .... | ..... | .... | ..... | .... | ..... | ..... |
| Ferrous sulfate | 0.02 | + | 0.1 | + | 0.05 | + | 0.02 | + | 0.01 | + |
| Iron and ammonium sulfate | 0.5 | + | 0.2 | + | 0.05 | + | ..... | .... | ..... | ..... |
| Cobalt sulfate | 0.05 | + | ..... | .... | ..... | .... | ..... | .... | ..... | ..... |
| Manganese sulfate | 0.2 | + | 0.1 | + | ..... | .... | ..... | .... | ..... | ..... |
| Mercuric sulfate | 1.0 | — | 0.25 | — | 0 | .... | ..... | .... | ..... | ..... |
| Ammonium sulfate | 0.5 | + | 0.2 | + | 0.1 | + | 0.05 | + | 0.02 | + |
| Zinc sulfate | 0.2 | + | 0.05 | + | 0.01 | + | 0 | .... | ..... | ..... |
| Aluminium and potassium sulfate | 0.5 | + | 0.05 | + | ..... | .... | ..... | .... | ..... | ..... |
| Barium sulfate | 10.0 | + | 5.0 | + | 2.0 | + | ..... | .... | ..... | ..... |
| Aluminium sulfate | 0.2 | + | 0.1 | + | ..... | .... | ..... | .... | ..... | ..... |
| Magnesium sulfate | 2.75 | + | 1.35 | + | 0.35 | + | 0.275 | + | ..... | ..... |
| Aluminium and ammonium sulfate | 0.5 | + | 0.1 | + | ..... | .... | ..... | .... | ..... | ..... |
| Checks | 18 tumblers. No nodules. | | | | | | | | | |

* + indicates organism present; — indicates organism not present.

### *In water cultures*

It has been shown that nodules are not produced in distilled water alone, nor, except in rare cases or in certain dilute concentrations, in distilled water to which the various salts of Pfeffer's solution have been added.

In order to test the ability of the organism to exist in these solutions without effecting inoculation, about 5 cubic centimeters of the liquid

from the various containers was used as a source of inoculating material for seedling host plants. The organism had been placed in the solution from four to six weeks previous to this determination. The containers for these solutions were covered thruout the entire period with paraffin paper, so that there was but little opportunity for the solutions to become contaminated.

The salts or combinations of salts tested with respect to their influence on the vitality of the organism are listed in table 15. The plus or the minus sign indicates the efficiency or the inefficiency of the organism to effect inoculation after remaining in the salt solution for from four to six weeks. In all cases the reinoculated plants grew for twenty-one days before examination.

TABLE 15. VITALITY OF BACILLUS RADICICOLA AFTER REMAINING FROM FOUR TO SIX WEEKS IN DISTILLED WATER TO WHICH HAD BEEN ADDED THE SALTS OF PFEFFER'S SOLUTION IN VARIOUS DILUTIONS AND COMBINATIONS*

| Substance used | Organism |
|---|---|
| Calcium nitrate | — |
| Potassium nitrate | + |
| Magnesium sulfate | + |
| Monopotassium phosphate | + |
| Potassium chloride | + |
| Ferric chloride | + |
| Calcium nitrate and potassium nitrate | + |
| Calcium nitrate and magnesium sulfate | + |
| Calcium nitrate and monopotassium phosphate | + |
| Calcium nitrate and potassium chloride | + |
| Calcium nitrate and ferric chloride | + |
| Potassium nitrate and magnesium sulfate | + |
| Potassium nitrate and monopotassium phosphate | — |
| Potassium nitrate and potassium chloride | + |
| Potassium nitrate and ferric chloride | + |
| Magnesium sulfate and monopotassium phosphate | — |
| Magnesium sulfate and potassium chloride | — |
| Magnesium sulfate and ferric chloride | + |
| Monopotassium phosphate and potassium chloride | + |
| Monopotassium phosphate and ferric chloride | — |
| Potassium chloride and ferric chloride | + |
| Calcium nitrate, potassium nitrate, and magnesium sulfate | + |
| Calcium nitrate, potassium nitrate, and monopotassium phosphate | + |
| Calcium nitrate, potassium nitrate, and potassium chloride | + |
| Potassium nitrate, magnesium sulfate, and monopotassium phosphate | — |
| Potassium nitrate, magnesium sulfate, and potassium chloride | + |
| Potassium nitrate, magnesium sulfate, and ferric chloride | + |
| Magnesium sulfate, monopotassium phosphate, and potassium chloride | + |
| Magnesium sulfate, monopotassium phosphate, and ferric chloride | + |
| Monopotassium phosphate, potassium chloride, and ferric chloride | + |
| Calcium nitrate, potassium nitrate, and ferric chloride | + |
| Calcium nitrate, potassium nitrate, magnesium sulfate, and monopotassium phosphate | + |
| Calcium nitrate, potassium nitrate, magnesium sulfate, and potassium chloride | + |

* + indicates organism present; — indicates organism not present.

TABLE 15 (*continued*)

| Substance used | Organism |
|---|---|
| Calcium nitrate, potassium nitrate, magnesium sulfate, and ferric chloride | + |
| Potassium nitrate, magnesium sulfate, monopotassium phosphate, and potassium chloride | + |
| Potassium nitrate, magnesium sulfate, monopotassium phosphate, and ferric chloride | + |
| Calcium nitrate, potassium nitrate, magnesium sulfate, monopotassium phosphate, and potassium chloride | + |
| Potassium nitrate, magnesium sulfate, monopotassium phosphate, potassium chloride, and ferric chloride | + |
| Calcium nitrate, potassium chloride, and ferric chloride | + |
| Calcium nitrate, monopotassium phosphate, and potassium chloride | + |
| Calcium nitrate, magnesium sulfate, and monopotassium phosphate | + |
| Potassium nitrate, potassium chloride, and ferric chloride | + |
| Potassium nitrate, monopotassium phosphate, and potassium chloride | + |
| Magnesium sulfate, potassium chloride, and ferric chloride | + |
| Calcium nitrate, potassium nitrate, potassium chloride, and ferric chloride | + |
| Calcium nitrate, potassium nitrate, monopotassium phosphate, and potassium chloride | + |
| Calcium nitrate, monopotassium phosphate, potassium chloride, and ferric chloride | + |
| Calcium nitrate, magnesium sulfate, monopotassium phosphate, and potassium chloride | + |
| Calcium nitrate, potassium nitrate, potassium chloride, and ferric chloride | + |
| Pfeffer's solution 8 concentration | + |
| Pfeffer's solution 4 concentration | — |
| Pfeffer's solution 3 concentration | + |
| Pfeffer's solution 1 concentration | — |
| Pfeffer's solution 1/2 concentration | + |
| Pfeffer's solution 1/4 concentration | + |
| Pfeffer's solution 1/8 concentration | + |
| Pfeffer's solution 1/16 concentration | + |
| Pfeffer's solution 1/32 concentration | + |
| Pfeffer's solution minus the nitrates, which were replaced by chlorides in equivalent amounts | |
| 8 concentration | + |
| 4 concentration | — |
| 2 concentration | — |
| 1 concentration | — |
| 1/2 concentration | — |
| 1/4 concentration | + |
| 1/8 concentration | + |
| 1/16 concentration | — |
| 1/32 concentration | + |
| Monopotassium phosphate in concentrations of | |
| 2 grams per liter | + |
| 1 gram per liter | + |
| 1/2 gram per liter | + |
| 1/4 gram per liter | + |
| 1/8 gram per liter | — |
| Magnesium sulfate in concentrations of | |
| 2 grams per liter | — |
| 1 gram per liter | — |
| 1/2 gram per liter | + |
| 1/4 gram per liter | + |
| 1/8 gram per liter | + |
| Total, 77. Organism present in 61 cases, not present in 16. | |

It will be observed from this list that the organism retained its vitality in 61 out of 77 cases, and that no one particular salt was present in all the 16 solutions in which the organism was not found.

*Conclusion*

These results lead to the conclusion that there is evidence for the belief that the chemical nature of the salt solution is a factor in controlling nodule production. In all these cases the organism, tho still living in the soil or water culture, was not capable of inducing nodule formation.

## TIME REQUIRED FOR INITIAL INFECTION

It is known that infection of the host plant by *Bacillus radicicola* takes place in a relatively short time, for plants that have been inoculated

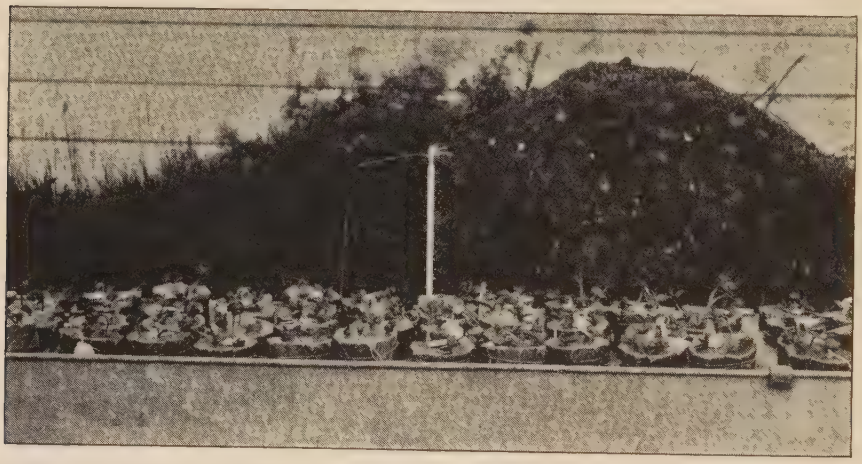

FIG. 94. METHOD OF TESTING FOR INITIAL PERIOD OF INFECTION, SHOWING SIZE OF PLANTS AT TIME OF TEST

with a pure culture will show abundant nodule development within ten days if the conditions are right. Peirce (1902) showed that on bur clover the characteristic bending of the root hairs previous to nodule formation will occur in twenty-four hours after inoculation. But the exact time required for the organism to penetrate the host, so far as the writer knows, has never been determined. Experiments were therefore made to ascertain this point.

Water cultures in tumblers of 250 cubic centimeters capacity were used. These cultures were set in water in a shallow pan and kept out of doors. Four soybean plantlets were inserted thru holes in the paper cover of each tumbler so that the roots were immersed. Water was run from the tap into the pan by means of a hose. Twenty-four hours later the contents of the tumblers were equally inoculated with a vig-

orously growing culture of *B. radicicola*. At certain intervals (as shown in table 16) two tumblers were removed from the pan and connected with siphons which carried fresh water into them, replacing the inoculated water. The rate of flow of the wash water was 250 cubic centimeters per 30 seconds, this being the volume of the tumbler. In all cases the siphons were kept running for a period not shorter than five days. After this washing the tumblers were returned to the pan, where a temperature of about 20° C. was being maintained. The contents of the tumblers were inoculated on June 29, 1914, and the roots were examined for nodules on July 14, 1914. The results are presented in table 16:

TABLE 16. TIME REQUIRED FOR INITIAL INFECTION

(Water culture, eight plants. Duration of experiment, 14 days. Temperature, 20° C.)

| Duration of contact between roots and inoculated water | Length of time roots were washed with running water from siphon (days) | Number of nodules that developed on roots as result of treatment |
|---|---|---|
| No contact | 6 | 0 |
| A few seconds | 6 | 1 |
| (Minutes) | | |
| 5 | 5 | 0 |
| 10 | 5 | 0 |
| 15 | 5 | 1 |
| 20 | 5 | 0 |
| 25 | 5 | 1 |
| 30 | 5 | 0 |
| 35 | 5 | 1 |
| 40 | 5 | 0 |
| 45 | 5 | 1 |
| (Hours) | | |
| 1 | 5 | 1 |
| 1¼ | 5 | 3 |
| 1⅔ | 5 | 0 |
| 2 | 5 | 13 |
| 2½ | 5 | 7 |
| 2¾ | 6 | 5 |
| 4 | 6 | 7 |
| 4⅔ | 6 | 19 |
| 5½ | 6 | 29 |
| 6½ | 6 | 14 |
| 10 | 6 | 23 |
| 12½ | 6 | 42 |
| 14 | 6 | 26 |
| 18 | 6 | 24 |
| (Days) | | |
| 14 | Not washed | 11 tumblers, 1145 (or 208 per 8 plants) |

From these data it is seen that infection occurred immediately, for washing did not remove all the organisms from the roots. The plant roots that were exposed for only a few seconds showed one nodule, and occasionally nodules were produced with the initial time of exposure under four and two-thirds hours. All nodules that were produced with an initial time of infection shorter than four and two-thirds hours appeared in the fork of the root system or in some place among the roots where the organisms would be protected from the water current of the siphon. The roots exposed for four and two-thirds hours before the siphon was started showed what might be called a general infection. By this is meant an infection of the root system in places freely exposed to the washing effect of the running water. It should be noted that there were not so many nodules produced at an exposure of eighteen hours as there were in cases in which the roots were not washed at all. This would be expected, for all infections that may occur do not necessarily occur in the first eighteen or twenty-four hours. In a previous determination to ascertain the time required for initial infection, in which the plants were considerably older when inoculated, general infection occurred with or after an exposure of five hours.

## IS NODULE FORMATION ASSOCIATED WITH THE NITROGEN REQUIREMENT OF PLANTS?

The views of various investigators imply that nodule formation is associated with a demand on the part of the plant for nitrogen. This view is dependent on the fact that nitrogenous compounds, and particularly nitrates, depress or inhibit nodule formation, and it has therefore been assumed that plants having a sufficient supply of nitrogen abstain from the production of nodules. This view suggests an ability on the part of the plant to preferentially adapt itself with respect to its nitrogen requirements. Such a view is not tenable in the light of present knowledge of plants. Furthermore, the various sulfates depress or inhibit nodule formation just as effectively as do the nitrates. It would appear that the respective explanations for the inhibiting action of nitrogenous compounds and of various sulfates on nodule formation are similar in nature. As an effective answer to the older views, as well as to determine whether the influences of the nitrogenous compounds are mainly local in their effect, the following experiments were made.

### *Experiment 1*

Thin-walled vessels were arranged side by side in pairs so that the roots of a single plant could be extended into the solutions in both containers. In order to have favorable conditions for nodule development, strontium chloride was added to one container in molecular concentration

1/5000. The other container received ammonium nitrate. The nitrate was varied in different containers, as is indicated in table 17. The root systems of young seedlings were divided, and one portion was extended into the nitrate solution and the other into the chloride solution. The volume of solution was the same for each half of the plant roots. After inoculating the contents of each container with a culture of *Bacillus radicicola* the plants were allowed to grow for fourteen days. This method offered to one-half of the root system all the nitrate that the plant could use, and permitted growth of a part of the root system in a solution containing only traces of nitrates. The results on nodule production are given in table 17:

TABLE 17. NODULE PRODUCTION ON INDIVIDUAL PLANTS WITH ROOTS DIVIDED, PART BEING PLACED IN A NITRATE SOLUTION AND THE REMAINDER IN A CHLORIDE SOLUTION*

(Fourteen days after inoculation. Strontium chloride, molecular solution 1/5000)

| Ammonium nitrate (molecular concentration) | Nodules on | |
|---|---|---|
| | Nitrate side | Chloride side |
| 1/5000 | + | + |
| 1/3500 | + | + |
| 1/2500 | + | + |
| 1/1250 | + | + |
| 1/800 | + | + |
| 1/600 | + | + |
| 1/500 | + | + |
| 1/450 | + | + |
| 1/300 | + | + |
| 1/250 | — | + |
| 1/220 | — | + |
| 1/185 | — | + |
| 1/175 | — | + |

* + indicates nodules present; — indicates no nodules.

It is brought out clearly in table 17 that nodule development occurred in every instance on the roots that grew in the chloride solution. It is also shown that the remainder of the root system, which was grown in the nitrate solution, produced nodules up to and including molecular concentration 1/300, and that the roots in greater molecular concentration than 1/300 produced no nodules.

*Experiment 2*

Young plants were grown in water culture to which was added ammonium nitrate in molecular concentration 1/1200. When the plants were

about eight to ten inches high, cotton was wrapped around each stem and twisted into a wick. This wick extended into a container holding a solution without nitrates. In a few days roots were produced which grew out into the cotton. When these roots appeared more nitrate was added to the water in which the primary root with its laterals was growing, until the concentration was molecular 1/250. This concentration is great enough to prevent nodule formation and still not great enough to noticeably injure plant growth. Both root systems were inoculated with *B. radicicola*, and after two weeks were examined for nodules.

The primary root and its laterals which were growing in the presence of the nitrate showed no nodule formation. The roots that were produced above the cotyledonary part in the cotton and subsequently inoculated, showed well-developed nodules.

### *Conclusion*

The data from these experiments show that the effect of the nitrates in depressing nodule formation is at most only local in character, and that the plant does not exercise any preference of methods for obtaining nitrogen.

## GENERAL DISCUSSION

The experiments described herein were undertaken in order to study the physiology of the causal organism of nodule formation on soybeans, and the factors influencing nodule development. The work was done under artificial conditions in the greenhouse and in the laboratory. The results obtained apply to this organism only, altho in many respects the data are in accord with those obtained by other investigators working with other strains of *Bacillus radicicola*. With the technique used it has been possible to demonstrate conclusively that the flagella of the soybean organism are peritrichous and that as many as four may be found. This peritrichous arrangement agrees with that found by De' Rossi (1907), Zipfel (1912), Kellerman (1912), and Prucha (1915), with the organism of other host plants. It would seem, therefore, that this character, as suggested by Kellerman and others, is uniform among the legume bacteria.

A review of the literature of the subject brings out very clearly the fact that nodule formation can be checked or stimulated, depending on the presence or the absence of certain salts and on the amount of moisture present. Additional light has been thrown on this point by extending the observations already made and by working under carefully controlled conditions. It is suggested that some of the discordant results reported in the literature are due to a lack of properly controlling many of the factors that bear directly or indirectly on nodule production. In this

connection one may refer to the soil moisture. In these experiments, with an increase of moisture content from 35 to 45 per cent the nodule production was more than doubled (table 12, page 396), while with an increase from 45 to 55 per cent it was nearly doubled.

To the class of compounds including those that stimulate nodule formation belong the chlorides, the phosphates, the calcium-containing compounds, and certain organic carbon compounds. Vines (1888–89) and others have suggested that nodules are produced only when the conditions of nutrition are favorable. Other investigators claim that the organism derives from the host plant carbohydrate material for its energy, and is thus attracted, and that nodule development will not occur unless the host plant is doing active photosynthetic work. While these and other suggested explanations as to the cause of nodule production may be of importance, they do not appear entirely satisfactory. It would seem in this connection that the application of sugars, or of such a material as starch, which probably serves as a source of energy for the organism and possibly for the plant, would furnish more nearly ideal conditions for the development of the organism than are found in the tissue of the host plant. But the data show that under such applications the nodule formation is more than trebled.

While certain compounds stimulate nodule formation, others just as effectively reduce it. In this class fall the nitrates, the ammonia-containing or -producing compounds, and the sulfates. These data suggest, therefore, that infection and subsequent nodule formation are inseparably combined with the protoplasmic activity. Whether this activity is manifest by the organism or by the host plant, or by both, has not been determined. Certain facts, however, have been established. In those cases in which the substances applied inhibited nodule formation, the organism remained alive and was capable of producing nodules immediately on being brought under favorable conditions. Further, if the action of these inhibiting substances resulted in a modification of the morphological or the physiological activities of the plant roots, the effect appeared locally and did not extend to other parts of the plant than those actually in contact with the inhibiting agent. Whether the reverse would be true of stimulative substances was not determined, but indications are in the affirmative. These results are significant from the standpoint of immunity; for if these substances render the plant immune to infection by *B. radicicola*, then this immunity also is only local and does not extend to other parts of the plant than those actually in contact with the immunizing substance; in other words, the plant does not absorb enough of this immunizing material and transfer it to all parts of the plant so that it can be used thruout as a means of protection.

The effect that such substances may have on the existence of the legume organism in the soil has been pointed out by Wohltmann (1902), who suggests that the addition of certain substances may in time destroy all the legume bacteria and that fertilizers should be chosen to avoid this condition. This may be important in certain cases, as Wohltmann has shown; but in other cases it may not be so important, for if two substances are added to the soil, one that promotes and one that inhibits nodule formation, their effect may be to neutralize each other.

It has been observed occasionally, also, that when the pure culture used in this work was used by farmers, negative results were obtained. Certain parts of these data may be cited to explain the failure, especially those obtained with lime in its various forms, for under its action the nodule production was more than trebled. The soil condition itself, by the application of fertilizing substances such as ammonium sulfate, potassium or sodium nitrate, or potassium or sodium sulfate, may have changed so that nodule formation could not occur. The data should be of especial significance to those southern States where high fertilizer applications are customary.

## SUMMARY

The chief points emphasized by this investigation are the following:

The causal organism of nodule production on soybean (*Soja max* Piper) is *Bacillus radicicola*. Its flagella are peritrichous, four being the largest number found. Its group number (which is a numerical system of recording the salient characters of an organism, and which was computed according to the card of the Society of American Bacteriologists for 1907) is B.222.3332033.

Of fifteen legumes grown in virgin Volusia silt loam soil, only one, *Trifolium pratense*, developed nodules without artificial inoculation.

The normal variation in nodule production under uniform conditions is probably not greater than ±12 per 100 plants, with an average of 3.59 per plant.

Of eighteen different nitrates added to Volusia silt loam soil in quantities appreciably below the amount necessary to cause injury to the plants, sixteen either reduced or completely inhibited nodule formation.

Twenty-two chlorides were tested in Volusia silt loam soil as to their effect on nodule production. The results were stimulative in all cases but five, in three of which the application was toxic, and in one the result was within the normal variation. Ammonium chloride, in the amount used, appreciably reduced nodule formation.

Some sulfates, such as sodium, magnesium, calcium, barium, zinc, and others, depressed nodule formation when applied to Volusia silt loam soil.

Phosphates in soil cultures increased nodule production. Their action was not so pronounced, however, as that of the chlorides.

Ammonia-containing or -producing compounds in most cases either reduced or entirely inhibited nodule production. When the ammonia was combined with a stimulating radical, its action was somewhat neutral.

Calcium compounds added to Volusia silt loam soil were in most cases effective in stimulating nodule formation. Calcium sulfate and calcium nitrate, however, reduced the number of nodules formed.

The carbonate radical showed no appreciable effect on nodule production.

Carbon-containing compounds, such as sugars, oxalic, lactic, and citric acids, tartrates, caffein, and cumarin, stimulated nodule production when in soil culture.

Moisture has an effect on nodule formation. As the moisture was increased from 25 per cent of the dry soil to 75 per cent, the nodule formation was correspondingly increased.

In distilled water cultures nodules were not produced except in rare cases, even tho various nutrient salts, in concentrations such as are used in Pfeffer's solution, were present singly or in combination. In distilled water which had been shaken with carbon black, nodule formation occurred only in rare cases. However, when the distilled water was shaken with carbon black and various salts in certain molecular concentrations were added to it, nodules appeared in appreciable numbers. This was especially true of the nitrates, the chlorides, and potassium and sodium sulfate.

Nitrates or sulfates applied to soil in quantities sufficient to inhibit nodule formation did not destroy *Bacillus radicicola* nor reduce its ability to produce nodules when brought under favorable conditions. Similar results were obtained with distilled water cultures alone and with cultures in distilled water to which were added nutrient salts singly or in combination.

The composition of the soil solution is a factor in controlling nodule production.

The time required for the legume organism to penetrate the tissue of the host plant is about five hours.

The inhibiting action of nitrogenous compounds on nodule formation is local in character.

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The bibliography appended is intended to cover most of the articles that deal incidentally or purposely with factors influencing nodule production; also, to lead readers to literature where descriptions of and opinions regarding *Bacillus radicicola* may be found. Many of these references have been an aid and an inspiration to the writer in directing the investigation.


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